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High prevalence of Hepatozoon spp. (Apicomplexa, hepatozoidae) infection in water pythons (Liasis fuscus) from tropical Australia

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Abstract

Molecular methods were used to identify blood parasites frequently observed in blood smears of water pythons (Liasis fuscus) captured in our study area in the Northern Territory of Australia. A nested polymerase chain reaction (PCR) using primers amplifying the 18s ribosomal RNA (rRNA) nuclear gene resulted in a short PCR product (180 bp) matching this region in the genus Hepatozoon. However, because of the short sequence obtained. 2 new primers were designed based on 18s rRNA sequences of 3 Hepatozoon taxa available in GenBank. Using these primers, approximately 600 bp of the parasite's 18s rRNA gene was amplified successfully and sequenced from 2 water python samples. The new primers were used to investigate the prevalence of blood parasites in 100 pythons. In 25 of these samples we did not observe any blood parasites when examining stained slides. All the samples revealed a 600-bp PCR product, demonstrating that pythons in which we did not visually observe any parasites were infected by Hepatozoon spp. We also analyzed the nucleotide sequences of blood parasites in 4 other reptile taxa commonly encountered in our study area. The sequences obtained from water pythons and from 1 of these taxa were identical, suggesting that the parasite is capable of infecting hosts at different taxonomic levels.

Keywords

tropical, liasis, water, australia, high, hepatozoidae, spp, hepatozoon, pythons, infection, apicomplexa, prevalence, fuscus

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High Prevalence of *Hepatozoon* Spp. (Apicomplexa, Hepatozoidae) Infection in Water Pythons (*Liasis fuscus*) From Tropical Australia

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ABSTRACT: Molecular methods were used to identify blood parasites frequently observed in blood smears of water pythons (*Liasis fuscus*) captured in our study area in the Northern Territory of Australia. A nested polymerase chain reaction (PCR) using primers amplifying the 18s ribosomal RNA (rRNA) nuclear gene resulted in a short PCR product (180 bp) matching this region in the genus *Hepatozoon*. However, because of the short sequence obtained, 2 new primers were designed based on 18s rRNA sequences of 3 *Hepatozoon* taxa available in GenBank. Using these primers, approximately 600 bp of the parasite's 18s rRNA gene was amplified successfully and sequenced from 2 water python samples. The new primers were used to investigate the prevalence of blood parasites in 100 pythons. In 25 of these samples we did not observe any blood parasites when examining stained slides. All the samples revealed a 600-bp PCR product, demonstrating that pythons in which we did not visually observe any parasites were infected by *Hepatozoon* spp. We also analyzed the nucleotide sequences of blood parasites in 4 other reptile taxa commonly encountered in our study area. The sequences obtained from water pythons and from 1 of these taxa were identical, suggesting that the parasite is capable of infecting hosts at different taxonomic levels.

In 1949, John Haldane initiated the idea that parasites could act as significant selective agents on their hosts, e.g., parasites could mediate competitive interactions between hosts, produce and maintain rare host genotypes, and even drive host speciation (Haldane, 1949). During the past decades Haldane's ideas have resulted in numerous publications focusing on parasite–host interactions. Parasites have been demonstrated to regulate host population size (Anderson and May, 1979), reduce host fecundity (Møller, 1993), increase host morbidity (weakness or other debility) (Clayton and Moore, 1997), and even cause host mortality (Coltman et al., 1999; Pampoule et al., 1999). At the genetic level parasites may structure genotypic polymorphism (Clark, 1979) and thus determine host genetic structure (Schykof and Schmid-Hempel, 1991). Furthermore, parasites are frequently invoked as a major factor driving the evolution of the tremendous complexity of the vertebrate immune system (Hedrick, 1994).

Since 1989, we have been conducting fieldwork on a large water python (*Liasis fuscus*) population living on the Adelaide River floodplain situated 60 km southeast of Darwin in the Northern Territory of Australia (Madsen and Shine, 2000). During the course of the study we discovered that the pythons are frequently infected by a wide range of gatstrointestinal and blood parasites that in numerous cases have resulted in death of the hosts (T. Madsen, pers. obs.). Thus, during the last 2 yr our work has focused on quantifying individual variation in parasite prevalence and developing methods to identify the hemogregarine parasites observed in python blood smears. However, relying only on scanning stained blood smears may mean that low levels of parasite infections will not be detected (Macdonald, 1926; Perkins and Martin, 1999). Therefore, in recent years molecular methods, both mitochondrial DNA, e.g., cytochrome *b*, and nuclear DNA, e.g., ribosomal RNA (rRNA), have been used increasingly to detect the presence and identity of hematozoan parasites (Escalante et al., 1998; Mathew et al., 2000; Perkins and Schall, 2002). In the present study we used polymerase chain reaction (PCR) and sequencing to identify and determine the taxonomic status of the blood parasites infecting the pythons and 4 other squamate taxa frequently encountered in our study area. We provide data on the prevalence of these parasites in the python population, and the possible pathogenic effects of the blood parasites on water pythons are discussed.

Water pythons are large, nonvenomous snakes that occur over a wide area of tropical Australia (Cogger, 1992). Blood samples (100 μl) were collected from 102 pythons, and genomic DNA was isolated from whole blood by phenol–chloroform extraction (Sambrook et al., 1989). Initially, we tested the set of primers, described by Li et al. (1995) and Perkins et al. (1998), amplifying the 18s rRNA nuclear gene of blood parasites. A nested PCR was performed according to the protocol of Perkins et al. (1998). The PCR product was resolved by electrophoresis on 2% agarose gel stained with ethidium bromide. The product was sequenced using the dye terminator cyclic sequencing (big dye) system on an ABI PrismTM 310 automated DNA sequencer (Perkin–Elmer, Applied Biosystems, Foster City, California). The amplification of the PCR product resulted in a sequence spanning approximately 180 bp. The sequence was sent to GenBank and matched the 18s rRNA gene region in *Hepatozoon* spp. by 97%. However, because of the short sequence obtained, we decided to design a new set of primers based on *Hepatozoon* spp. 18s rRNA gene sequences available from GenBank. The sequences of 3 taxa were aligned; *H. catesbeiana* (AF176837), *H. canis* (AF176835), and *H. americanus* (AF176836) and the conservative regions were used to design 2 new primers: HepF300: 5′-GTT TCT GAC CTA TCA GCT TTC GAC G-3′, Hep900 5′-C AAA TCT AAG AAT TTC ACC TCT GAC-3′.

The PCR reactions were run in a 50-μl reaction mixture containing 50 ng total genomic DNA, 1 U of AmpliTaq polymerase (Perkin–Elmer, Applied Biosystems), 1.5 mM $MgCl_2$, 0.125 mM of each nucleotide, 5 μl of Perkin–Elmer GeneAmp 10× PCR buffer (100 mM Tris–HCl, 500 mM KCl, and 0.01% gelatine), and 0.6 μM of each primer. The reaction mixture was heated to 94 C for 3 min, and then amplification was performed through 35 cycles at 94 C for 30 sec, 60 C for 30 sec, and 72 C for 1 min. After the 35 cycles there was a final 10-min extension at 72 C. The PCR products of the 2 water pythons were resolved and sequenced as described above.

Perkins and Martin (1999) demonstrated that 18s rRNA primers may amplify host DNA and thus give rise to false-positive results. The following 3 steps were undertaken to reduce this risk: (1) to avoid contamination, negative controls were run with each set of reactions, using 1 μl of sterile Milli-Q water in place of the template (all other reagent concentrations remained the same); (2) our primers were submitted to Genbank, and the alignment demonstrated that our primers matched

only the 18s rRNA gene of *Hepatozoon* spp.; and (3) DNA of 2 great reed warblers (*Acrocephalus arundinaceus*) known to be infected with both *Plasmodium* spp. and *Haemoproteus* spp. were amplified, but both amplifications failed to reveal any PCR products. These results suggest strongly that our primers amplified only the 18s rRNA gene in *Hepatozoon* spp.

The new primers were used to investigate the prevalence of blood parasites in 100 pythons (none of these samples was sequenced). In 25 of these samples we had not observed any blood parasites when examining stained blood smears (2,000 erythrocytes were counted on each slide).

The primers were also used to investigate whether the 4 other squamate taxa frequently encountered in our study area were infected by *Hepatozoon*. We sampled 1 brown tree snake (*Boiga irregularis*), 2 slaty-grey snakes (*Stegonotus cucullatus*), 2 spotted tree goannas (*Varanus scalaris*), and 2 northern sand goannas (*V. panoptes*).

In all the 100 water pythons examined, we obtained a PCR product spanning approximately 600 bp. Thus, the 25 pythons in which we did not detect any parasites when scanning stained blood smears were infected by *Hepatozoon* spp., confirming that relying only on visual methodology may indeed produce false negatives. Furthermore, all the 7 specimens of the other 4 taxa revealed a similar PCR product, demonstrating that these squamates were also infected by *Hepatozoon* spp.

The 581-bp parasite sequences obtained from the 2 water pythons and the 2 northern sand goannas were identical. Pairwise comparisons (Kimura, 1980) demonstrated that all the 9 *Hepatozoon* haplotypes from the 5 host species were very similar (genetic distance ranging from 0.000 to 0.029), compared with the haplotypes of amphibian and canid *Hepatozoon* parasites (*H. catesbianae, H. americanum*, and *H. canis*; pairwise genetic distance comparisons ranged from 0.047 to 0.073). The sequences of the 5 host species have been deposited in GenBank (accession numbers: AY252103, AY252104, AY252105, AY252106, AY252107, AY252108, AY252109, AY252110, AY25211).

Hemogregarines of the Hepatozoidae have been reported to be the most common group of intracellular protozoan blood parasites found in snakes (Telford, 1984; Wozniak et al., 1996). These findings are supported by the results from the present study. Furthermore, the prevalence of *Hepatozoon* spp. infections of squamate reptiles in our study area appears to be very high because all the 100 water pythons and all the 7 specimens of the 4 other squamate taxa were infected.

On the basis of morphological studies, Telford et al. (2001) suggested that different *Hepatozoon* taxa often exhibit high levels of host specificity. However, our results demonstrate that *Hepatozoon* spp. exhibit identical nucleotide sequences, which indicates that the parasites are probably in the same taxon and are able to infect not only different host species but also different squamate families, i.e., Boidae and Varanidae.

Several workers have suggested that hemogregarine parasites exhibit low pathogenetic effects on their reptilian hosts (Nadler and Miller, 1984; Wozniak et al., 1994). However, blood parasite infections may cause leukocyte and erythrocyte dysfunction, with the host becoming more susceptible to systemic infections (Smith et al., 1999; Inokuma et al., 2002). Furthermore, our results suggest that *Hepatozoon* spp. with identical nucleotide sequences are able to infect different host organisms, suggesting that host shifts may occur among some of the squamate taxa found in our study area. Such host shifts have been reported in association with a change in parasite virulence (Toft and Karter, 1990). The findings by Toft and Karter (1990) were supported by Wozniak et al. (1996), who showed that lizards previously unexposed to *Hepatozoon* spp. suffered severe pathological effects when infected with *H. mocassini*. Another example of increased virulence caused by host shifting is the severe pathology, often fatal, associated with infection by *H. americanum* in dogs, as reported by Baneth et al. (2003). Our preliminary analyses suggest that *Hepatozoon* spp. may exert a significant effect on water python growth rates, i.e., high infection levels are significantly correlated with reduced growth rates as compared with snakes with low parasite numbers (T. Madsen, pers. obs.). Thus, the possible pathogenic effect of these hemogregarines may play a complex role in the evolution of their reptilian hosts; this potential phenomenon warrants further research.

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LITERATURE CITED

ANDERSON, R. M., AND R. M. MAY. 1979. Population biology of infectious diseases. Nature **280:** 361–366.

BANETH, G., J. S. MATHEW, V. SCHKAP, D. K. MACINTIRE, J. R. BARTA, AND S. A. EWING. 2003. Canine hepatozoonosis: Two disease syndromes caused by separate *Hepatozoon* spp. Trends in Parasitology **19:** 27–31.

CLARK, B. C. 1979. The evolution of genetic diversity. Proceedings of the Royal Society of London B Biological Sciences **205:** 453–474.

CLAYTON, D. H., AND J. MOORE. 1997. Introduction. *In* Host-parasite evolution, general principles and avian models, D. H. Clayton and J. Moore (eds.). Oxford University Press, Oxford, U.K., p. 1–6.

COGGER, H. 1992. Reptiles and amphibians of Australia. Reed Books, Sydney, Australia, 688 p.

COLTMAN, D. W., J. G. PILKINGTON, J. A. SMITH, AND J. M. PEMBERTON. 1999. Parasite mediated selection against inbred soay sheep in a free-living, island population. Evolution **53:** 1259–1267.

ESCALANTE, A. A., D. E. FREELAND, W. E. COLLINS, AND A. A. LAL. 1998. The evolution of primate malaria parasites based on the gene encoding cytochrome *b* from the linear mitochondrial genome. Proceedings of the National Academy of Sciences of the United States of America **95:** 8124–8129.

HALDANE, J. B. S. 1949. Disease and evolution. La Ricerca Scientica, Supplement **19:** 68–76.

HEDRICK, P. W. 1994. Evolutionary genetics of the major histocompatibility complex. The American Naturalist **143:** 945–964.

INOKUMA, H., M. OKUDA, K. OHNO, K. SHIMODA, AND T. ONISHI. 2002. Analysis of the 18 rRNA gene sequence of *Hepatozoon* in two Japanese dogs. Veterinary Parasitology **106:** 265–271.

KIMURA, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution **16:** 111–120.

LI, J., R. A. WIRTZ, G. A. MCCONKEY, J. SATTABONGKOT, A. P. WATERS, M. J. ROGERS, AND T. F. MCCUTCHAN. 1995. Plasmodium: Genus-conserved primers for species identification and quantification. Experimental Parasitology **81:** 182–190.

MACDONALD, G. 1926. Malaria in children of Freetown, Sierra Leone. Annals of Tropical Medicine and Parsitology **20:** 239–262.

MADSEN, T., AND R. SHINE. 2000. Silver spoons and snake sizes: Prey availability early in life influences long-term growth rates of free-ranging pythons. Journal of Animal Ecology **69:** 952–958.

MATHEW, J. S., R. A. VAN DEN BUSCHE, S. A. EWING, J. R. MALAYER, B. R. LATHA, AND R. J. PANCIERA. 2000. Phylogenetic relationships of *Hepatozoon* (Apicomplexa: Adeleorina) based on molecular, morphologic and life-cycle characters. Journal of Parasitology **86:** 366–372.

MØLLER, A. P. 1993. Parasitism and the evolution of host life history. *In* Host-parasite evolution, general principles and avian models, D. H. Clayton and J. Moore (eds.). Oxford University Press, Oxford, U.K., p. 105–127.

NADLER, S. A., AND J. H. MILLER. 1984. A redescription of *Hepatozoon mocassini* (Laveran, 1902) n. comb. from *Agkistrodon piscivorus* Troost, 1836. Journal of Protozoology **31:** 321–324.

PAMPOULE, C., S. MORAND, A. LAMBERT, E. ROSECCHI, J. L. BOUCHEREAU, AND A. J. CRIVELLI. 1999. Influence of the trematode *Aphalloides coelomica* Dollfus, Chabuad and Golvan, 1957 on the fecundity and survival of *Pomatoschitus microps* (Krøyer, 1838) (Teleostei: Gobiidae). Parasitology **119:** 61–67.

PERKINS, S. L., AND J. M. MARTIN. 1999. Conserved polymerase chain reaction primers fail in diagnosis of parasite infections. Journal of Parsitology **85:** 982–984.

———, S. M. OSGOOD, AND J. SCHALL. 1998. Use of PCR for detection of subpatent infections of lizard malaria: Implications for epitozoology. Molecular Ecology **7:** 1587–1590.

———, AND J. J. SCHALL. 2002. A molecular phylogeny of malarial parasites recovered from cytochrome *b* gene sequences. Journal of Parasitology **88:** 972–978.

SAMBROOK, J., E. F. FRITSCH, AND T. MANIATIS. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

SCHYKOF, J. A., AND P. SCHMID-HEMPEL. 1991. Genetic relatedness and eusociality: Parasite mediated selection on the genetic composition of groups. Behavioural Ecology and Sociobiology **28:** 371–376.

SMITH, T. G., B. KIM, AND S. S. DESSER. 1999. Phylogenetic relationships among *Hepatozoon* species from snakes, frogs and mosquitoes of Ontario, Canada, determined by ITS-1 nucleotide sequences and life-cycle, morphological and developmental characteristics. International Journal for Parasitology **29:** 293–304.

TELFORD, S. R. JR. 1984. Reptilian hemoparasites. *In* Diseases of amphibians and reptiles, G. L. Hoff, F. L. Frye, and E. R. Jacbson (eds.). Plenum Publishing Corporation, New York, p. 385–517.

———, E. J. WOZNIAK, AND J. F. BUTLER. 2001. Haemogregarine specifity in two communities of Florida snakes: With descriptions of six new species of *Hepatozoon* (Apicomplexa: Hepatozoidae) and a possible species of *Haemogregarina* (Apicomplexa: Haemogregarinidae). Journal of Parasitology **87:** 890–905.

TOFT, C. A., AND A. J. KARTER. 1990. Parasite–host coevolution. Trends in Ecology and Evolution **5:** 326–329.

WOZNIAK, E. J., K. R. KAZACOS, S. R. TELFORD JR., AND G. L. MCLAUGHLIN. 1996. Characterization of the clinical and anatomical pathological changes associated with *Hepatozoon mocassini* infections in unnatural reptilian hosts. International Journal for Parasitology **26:** 141–146.

———, G. L. MCLAUGHLIN, AND S. R. TELFORD JR., 1994. Description of the vertebrate stages of a hemogregarine species naturally infecting Mojave Desert sidewinders (*Crotalus cerastes*). Journal of Zoo and Wildlife Medicine **25:** 103–110.

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